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Development of Fermented Whey Beverage Supplemented with Lactic Acid Bacteria and *Plumbago Zeylanica* Extract

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ABSTRACT

The global demand for "healthy" food and drinks in the growing market endorses the old diet practise of nutritional needs and the preventive functioning of diseases by immune boosting. Beverages in particular are by far the most active functional group. Therefore, this study emphasizes on production of functional fermented whey-based herbal beverage. The beverage was prepared with whey, citric acid (0.3%), sugar (7%), orange flavour (0.02%) and fermented using *Lactobacillus* strains (1% of *Lactobacillus fermentum* KGL4 and *Lactobacillus plantarum* KGL3A in 1:1 ratio) and *Plumbago zeylanica* extract was added considering its therapeutic properties. Further storage studies with various parameters (pH, TA (titratable acidity), microbial, anti-oxidative and antibiotic activity) were conducted on the basis of sensory analysisin room as well as refrigerated condition in every 5 day of interval for 20 days.The study shows highest scores in pH (4.316±0.037) at 25^{0}C in day 0, TA (1.821±0.027) at 25^{0}C in day 10, microbial (10.549 ±0.032) at 25^{0}C in day 5, antioxidative (89.04 ± 3.32) at 6 ^{0}C in day 0 after 10 min, antibiotic (40.00±0.00, in tetracycline and rifampicin) at 6^{0}C in day 20. Throughout the study increasing in TA, microbial and antibiotic susceptibility with decreasing in pH, antioxidative and microbial count was noted. However, the results revealed that whey beverages had the highest functional properties and accessibility when consumed in refrigerated conditions till day 15.

Key words : Fermented whey beverage, Lactic acid bacteria, *Plumbago zeylanica.*

INTRODUCTION

Over the last few decades, there has been an increase in demand for "healthy" foods and beverages in many parts of the world (Ozen *et al.* 2012). In recent times the reliance on synthetics has comparatively diminish and people are relying on the naturals with a hope for protection and security (Sun-Waterhouse, 2011).*Plumbago zeylanica* commonly known as "White lead worth" in English and "Chitrak" in Sanskrit is one such herb that is widely available in the Garo hills of Meghalaya and belongs to the Plumbaginaceae family. It has enormous therapeutic values with variety of bioactive compounds such as

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napthoquinons, flavonoids, alkaloids, glycosides, steroids, fats, proteins and tannins,which can also be an effective food preservative (Singh and Kumar, 2019).Whey is composed of approximately 85–90% of the milk volume, constituting lactose (5%), water (93%), minerals (0.5%), proteins (0.85%) and a residue of fat (0.36 %) (Pescuma *et al*.2008). Numerous attempts have been made over the years to convert large quantities of whey produced as a by-product of the channa and paneer industries into a suitable food product (Djurić *et al.* 2004). One of the most appealing ways to use whey for human consumption is to turn it into a beverage by combining it with distinct food ingredients (Satpute *et al*. 2018). Despite the fact that whey proteins have a variety of beneficial properties, one of its major β-lactoglobulin (BLG) enzymes is the most common dairy allergen which can be conquered by probiotic bacteria such as, Lactic Acid Bacteria (LAB) which have the ability to hydrolyze milk proteins and degrade BLG during the whey and milk development processes (Pescuma *et al.* 2008).Probiotics are currently defined as "live microorganisms that confer a health benefit to the host when administered in adequate amounts"(Saarela. 2009). Fermentation is one of the oldest food preservation and biological improvement methods for dairy by-products into value added products (Khedkar *et al*. 2014).The growth of whey beverage production via fermentation with probiotic bacteria is given special attention so the most important step is to choose the appropriate bacterial culture for the production of a functional beverage with a high nutritional value and suitable sensory characteristics (Jeličić *et al.* 2008). LAB has the ability to ferment lactose into lactic acid present in whey which gives new flavours and has the potential to inhibit spoilage pathogens (Khedkar *et al*. 2014).This research has therefore primarily been concerned with the fortification of herbal plant extracts and potential probiotics in whey-based beverages. A functional whey beverage was prepared using *Plumbago zeylanica* and LAB infusion and their bio-functional properties have been assessed.

MATERIALS AND METHODS

Sample collection, materials and bacterial strains

Chitrak (*Plumbago zeylanica*) leaves and fresh cow milk were collected from the RDAP farm, NEHU, Tura Campus. Other ingredients like orange flavour and sugar have been purchased from the local market of Tura. Methanol was purchased from Merck Specialities Pvt., Ltd., India. A set of LAB strains, *Lactobacillus fermentum* KGL4 (NCBI Gen Bank Accession No. MF951099) and *Lactobacillus plantarum* KGL3A (NCBI Gen Bank Accession No. MG722814) were procured from Animal Science Laboratory of Department of RDAP, NEHU Tura Campus (Hati *et al.* 2019).

Preparation of methanolic plant extract: Fresh leaves of *Plumbago zeylanica* were washed, crisp dried and reduced to a coarse powder in an electrical blender. Powdered sample (25g) were soaked in 100 ml methanol (80 per cent v/v) separately in air tight reagent bottle for 3 days at 30 on a rotator shaker (100 RPM) (AIT-121, ACMAS Technologies). The methanol infusions were filtered using muslin cloth and filter paper (Whatman No.1) then further concentrated in hot air oven at 40^0c. The dry weights of *Plumbago zeylanica* extracts were taken and dissolved in distilled water to obtain solutions of the plant extract (5 mg/ml) which were filter sterilised using Millipore filter (0.45 µm, HiMedia) for further use (Murugan *et al.* 2018).

Preparation of the whey beverage: Fresh cow milk was collected and boiled in a stainless steel vessel at 80 ^{0}c and milk was coagulated using 0.2% citric acid, followed by gently stirring until the coagulation of milk was visible. Whey and solid mass were allowed to cool at room temperature and filtered using muslin cloth (Dhamsaniya and Varshney, 2013). The obtained whey was further processed by addition of sugar (7%), followed by addition of *Plumbago zeylanica* extract as per treatment combination 1%, 3%, 5%. The prepared herbal whey beverage was filtered with the help of filter paper (Whatman No.1) (Satpute *et al.* 2018). Herbal whey was heated at 85-90^0C for 10 mins and let to cool down to 42^0C. The whey was then inoculated at different rates (1%, 2% and 3%) in 1:1 ratio of the Lactobacillus strains. The obtained whey was added with orange flavour to mask the off flavour and aroma in the taste of the obtained whey (Burrington K.J. 2012) and incubated for 24h at 37^0C. The fermented herbal whey beverage was bottled in

a sterile glass container and stored at refrigerated (6^0C) as well as at room temperature (25^0C) (Khedkar *et al.* 2014).

Sensory evaluation

The sensory evaluation of the product was carried out for the different treatments (1%, 3% and 5%) of *Plumbago zeylanica* extract with respect to different batches (1%, 2%, 3%) of *Lactobacillus* strains. It was accessed by 5 panellists comprising of research scholars, office staff and faculty between the age range of 25-40 years from the Department of RDAP, NEHU, Tura Campus. Nine-point Hedonic scale was used to assess the analysis following Khupse *et al.* (2019) with some modification. The best compatible treatment was selected on the basis of sensory analysis where, 5% concentration of *Plumbago zeylanica* extract and 1% concentration of *Lactobacillus* strains was selected and further shelf-life studies has been performed on the same in 6^0C as well as 25^0C for different storage days.

Shelf-life study of the fermented whey beverages

Shelf-life study procedure was performed in accordance with Yadav *et al*. (2010) with minor modifications; the product was stored in 6^0C as well as 25^0C for 20 days. The samples were accessed for their pH, titratable acidity, microbial analysis, sensory evaluation and bio-functional evaluation (antioxidative and antibiotic activity) for every 5 days' interval.

Analytical tests

pH and titratable acidity: pH of the product was determined and titratable acidity was evaluated as per the method described by Gorachiya *et al.*(2018).

Microbial analysis: The product has been assessed by the methods described by Ismail *et al.* (2011) with slight modifications. The total number of colonies was determined as per log (CFU/ml) (Abraham *et al*. 2014).

Bio-functional evaluations: The ABTS (2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical-scavenging activity was performed for determination of antioxidative activity according to the methods described by Shalaby and Shanab (2013). Antibiotic activity of the product was determined by Kirby-Bauer disc diffusion method using de Mann, Rogosa and Sharpe (MRS) agar (HiMedia, India) to ten clinically relevant antibiotic discs (Kanamycin 30, Nalidixic acid 30, Vancomycin 30, Azithromycin 15, Methicillin 15, Tetracycline 30, Streptomycin 10, Oxacillin 1, Erythromycin 15 and Rifampin 5).An antibiotic zone scale was used to verify susceptibility where the diameters (mm) of the inhibition zone around the antibiotic discs were estimated for the sample as shown in (Table 3). All the evaluation was done at an interval of 5 days during the storage period of 20 days under 6^0C as well as at 25^0C.

Statistical analysis

Three independent replicates have been carried out for the study and the results for the experiment were expressed as mean ± standard deviation (SD). Data were evaluated using one-way and two-way analysis of variance (ANOVA) and comparative study was made through Tukey's test with a least significant difference ($p \leq 0.05$) using IBM SPSS statistical program version 23.

RESULTS AND DISCUSSION

Sensory analysis:The sensory analysis of the product was assessed by the above-mentioned method and results had been depicted in (Table 1). By ANOVA and Tukey's test it was found that the product stored at 6 ^{0}C and 25 ^{0}C differ significantly from each other ($p \leq 0.05$) at all storage days and all sensory parameters. Panellists have always favoured the 6□ product over 25^0C which was reported to be significantly deteriorating during the storage days. At 6^0C, colour and appearance on day 10 with 7.56 ± 0.62, flavor on day 15 with 7.66 ± 0.61, body (consistency) on day 10 with 7.56 ± 0.62, product acidity on day 10 with 7.33± 0.81 and overall acceptability on day 10 with 7.80 ± 0.41scored highest among all days. The product has been accepted by the panellist till day 15 with uniformly maintained scores throughout the shelf life except for acidity and body consistency on day 20. (Yadav *et al*. 2010) developed a whey based banana herbal beverage incorporating with 2% *Mentha*

Table 1. Microbial analysis of orange flavoured whey-based herbal beverage during (20 days) storage study. Mean ± SD values (n = 3). Tukey's test at $p \leq 0.05$ demonstrates that values in each cell with different superscripts differ significantly from each other. The alphabets in small and CAPITALS letters were used to distinguish within different storage days in 25^{0}C and 6^{0}C respectively. The highest activity in the concerned groups is represented by shaded cells. '-': product has been discarded by the panellist.

Storage Days	Lactobacillus count (log CFU/ml)		Yeast and mold count (log CFU/ml)		Coliforms count (log CFU/ml)	
	Storage temp. (6^{0}C)	Storage temp. (25^{0}C)	Storage temp. (6^{0}C)	Storage temp. (25^{0}C)	Storage temp (6^{0}C)	Storage temp (25^{0}C)
Day 0	8.913 ± 0.009^{A}	9.365 ± 0.069^{a}	Absent in 1 ml	Absent in 1 ml	Absent in 1 ml	Absent in 1 ml
Day 5	10.414±0.034^{B}	10.549 ±0.032^{b}	Absent in 1 ml	Absent in 1 ml	Absent in 1 ml	Absent in 1 ml
Day 10	9.893 ± 0.041^{C}	10.180 ±0.014^{c}	Absent in 1 ml	6.453 ± 0.049	Absent in 1 ml	Absent in 1 ml
Day 15	9.865 ± 0.030^{C}	-	Absent in 1 ml	-	Absent in 1 ml	-
Day 20	9.361 ± 0.013^{D}	-	6.483 ± 0.040	-	8.343 ± 0.034	-

extract, reported similar results with respect to acidity and acceptability of the product viz., acceptable till day 15 KG *et al*. (2016) and Khupse *et al*. (2019) also obtained similar results with respect to the sensory analysis of the product.

pH and titratable acidity: The mean pH values of the product decreased from 4.240±0.036 on day 0 to 3.536±0.0 20 on day 20 at 6 ^{0}C with respect to storage days ($p \leq 0.05$). However, the panellist accepted the product at 25^{0}C until day 10. Decline in pH during storage is observed which may be due to the action of citric and ascorbic acid on the sugar and protein component of the product. Production of organic acids and amino acids lead to an increase in acidity thereby a decrease in pH, as also reported for mango based beverages (Gorachiya *et al.* 2018). The titratable acidity of the product stored at 6 ^{0}C for day 0 differs ($p \leq 0.05$) from day 10, 15 and 20, showing highest score at day 20 (1.166±0.024). However, increasing acidity in the product was observed with respect to storage days in both the storage condition. This might occur due to fermentation effect (Saha *et al*. 2017). Similar findings were observed with significantly increased titratable acidity by Babar *et al.* (2008) in the analysis carried out for the use of pomegranate juice in the preparation of channa whey.

Table 2. Antioxidative activity (%) of orange flavoured whey-based herbal beverage during (20 days) storage study. Mean ± SD values (n = 3). Tukey's test at $p \leq 0.05$ demonstrates that values in each cell with different superscripts differ significantly from each other. The highest activity in the concerned groups is represented by shaded cells. The alphabets in small and CAPITALS letters were used to distinguish within different storage days in 25^{0}C and 6^{0}C respectively.

Days	Storage temp. 6°C		Storage temp. 25°C	
	Initial (0 seconds)	Final (600 seconds)	Initial (0 seconds)	Final (600 seconds)
0	64.90±5.36^{A}	89.04±3.32^{C}	53.14±4.49^{a}	81.42±2.64^{d}
5	57.23±3.05^{A}	84.38±3.75^{CD}	43.95±5.09^{b}	80.95±3.59^{d}
10	48.00±3.70^{B}	81.95±2.21^{CDE}	23.85±1.78^{c}	66.76±1.76^{e}
15	47.61±1.21^{B}	79.90±3.87^{DE}	-	-
20	44.85±3.80^{B}	73.57±3.46^{F}	-	-

Microbial analysis:The microbial evaluation of product as shown in (Table. 2) was performed by analysing the LAB, yeast & moulds and coliforms count during the course of shelf-life study. A total increasing of LAB has been observed and over the period of (20 days) storage it increases from day 0 (8.913 ± 0.009 log CFU/ml in 6^{0}C) today 20 (9.361 ± 0.013 log CFU/ml in 6^{0}C) (Table 2). However, at 25^{0}C yeast & mould was observed on day 10 (6.453 ± 0.049) due to which further analysis was suspended. By ANOVA and Tukey's test it showed that the LAB count of the product stored at 6 and 25^{0}C differ significantly from each other ($p \leq 0.05$). Coliforms was absent throughout the shelf life in both the storage condition. However, even after 20 days of 6^{0}C storage the total viable count of > 106 CFU/ml was maintained which according to international guidelines is mandatory for probiotic product (Thakkar *et al*. 2018). In the study of Tootoonchi *et al.* (2015), L. *acidophilus* encapsulated in orange juice and Yonis *et al.* (2014) on whey guava drinks similar results had been observed. Yeast and mould counts were detected in day 10 at 25^{0}C which was similar to the results of Gorachiya *et al.* (2018). Throughout the study no

Table 3. Antibiotic susceptibility of whey-based herbal beverage. K30=Kanamycin, NA30=Nalidixic acid, VA30=Vancomycin, AZM15=Azithromycin, MET15=Methicillin, TE30=Tetracycline, S10=Streptomycin, OX1=Oxacillin, E15=Erythromycin, RIF5=Rifampicin; mm= diameter of zone of inhibition around the antibiotic discs; ZOI= zone of inhibition; * = Not Detected; R= Resistant (0 to 15 mm), I= Intermediate (15.01 to 20.00), S= Sensitive (more than 20 mm), as per the guidelines of Clinical and Laboratory Standards Institute, 26th Edition. Each sample has three independent determinations (n = 3), represented in mean ± SD values. Tukey's test at $p \leq 0.05$ demonstrates that values in each cell with different superscripts differ significantly from each other. The alphabets in small and CAPITALS letters were used to categorise antibiotic susceptibility at 6^0C and 25^0C storage conditions.The highest activity in the concerned groups is represented by shaded cells. '-': product has been discarded by the panellist.

Days	Storage temp	Antibiotics (Conc. in µg) ZOI in mm									
	(^{0}C)	AZM15	VA30	TE30	OX1	RIF5	NA30	E15	MET15	K30	S10
0	6	27.33 ± 2.30^{A}	R	38.33 ± 2.88^{B}	R	38.33 ± 2.08^{B}	R	30.67 ± 1.52^{A}	R	R	13.67 ± 1.15^{C}
	25	28.33 ± 2.51^{a}	R	37.33 ± 2.51^{b}	R	37.67 ± 1.52^{b}	R	35.00 ± 2.00^{b}	R	R	17.33 ± 0.57^{c}
5	6	28.00 ± 0.00^{C}	R	38.33 ± 0.57^{D}	R	37.00 ± 1.73^{D}	R	33.67 ± 0.57^{E}	R	R	14.00 ± 1.00^{F}
	25	26.67 ± 3.05^{d}	R	38.33 ± 0.57^{e}	R	36.67 ± 0.57^{ef}	R	32.67 ± 0.57^{f}	R	R	13.33 ± 1.15^{g}
10	6	30.00 ± 2.00^{G}	R	38.67 ± 1.15^{H}	R	38.00 ± 2.00^{H}	R	31.67 ± 2.08^{G}	R	R	19.00±2.64I
	25	*	R	*	R	*	R	*	R	R	*
15	6	31.00 ± 0.00^{J}	R	36.00 ± 1.00^{K}	R	38.67 ± 1.15^{L}	R	34.67 ± 1.15^{K}	R	R	11.00 ± 0.00^{M}
	25	-	R	-	R	-	R	-	R	R	-
20	6	31.33 ± 1.15^{N}	R	40.00 ± 0.00^{O}	R	40.00 ± 0.00^{O}	R	38.67 ± 1.15^{O}	R	R	15.00 ± 1.73^{P}
	25	-	R	-	R	-	R	-	R	R	-

coliform counts were detected for both the storage condition, which can be concluded that the product is free of faecal contamination.

Antioxidative activity (AOA): TheAOA of the product was shown in (Table. 3) was performed using ABTS radical scavenging assay. A wide range of antioxidant capacity was observed at 6^0C as well as 25 ^{0}Cduring different storage periods. By ANOVA and Tukey's test it has been observed that the results differ significantly ($p\leq0.05$) with respect to storage condition, in all storage days as well as initial and

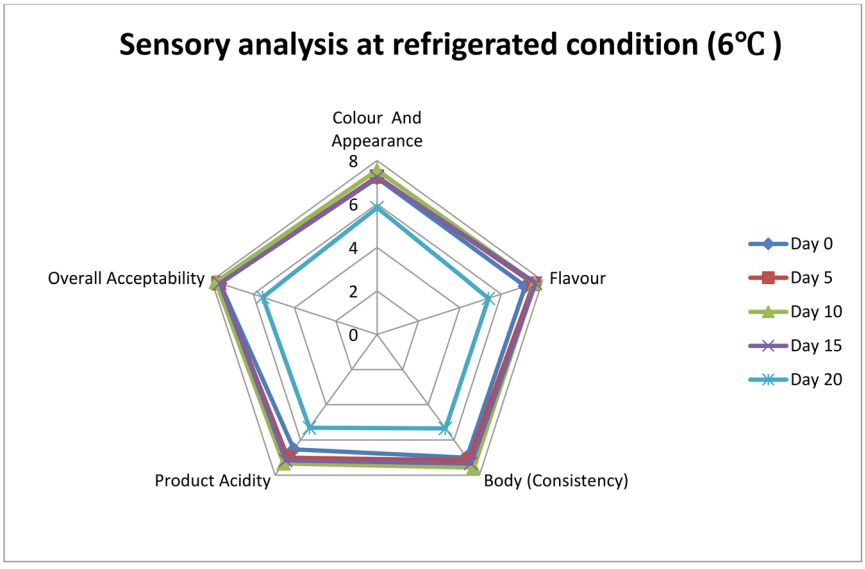

Fig 1. Sensory analysis of whey-based herbal beverage under refrigerated condition (6^0C) at different storage days

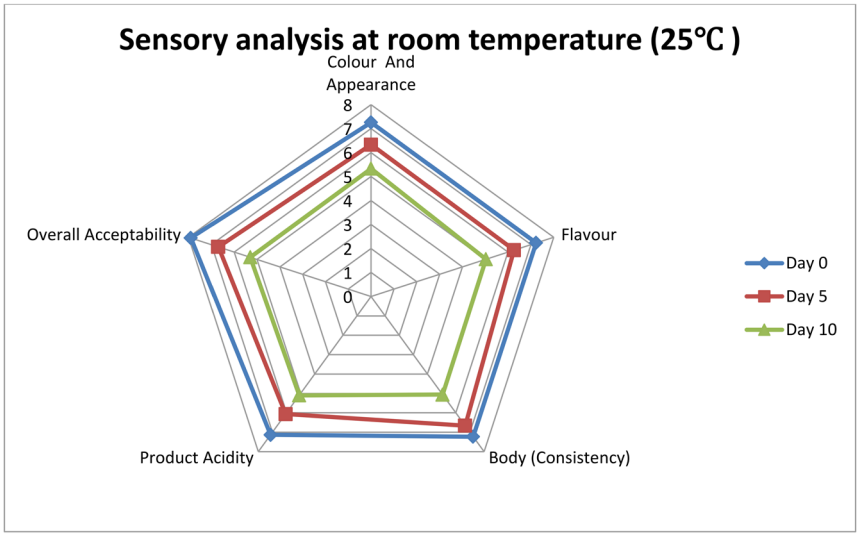

Fig 2. Sensory analysis ofwhey-based herbal beverage under room temperature (25^{0}C) at different storage days.

final readings except for day 5 final readings in both the storage conditions. A wide range of antioxidant capacity was observed at 6°C as well as 25°C during different storage periods. Among them, 6°C showed the highest antioxidative activity on Day 0 (64.9% at 0 min to 89.04 % at 10 min), followed by day 5 (57.23% at 0 min to 84.38% at 10 min). In our study, Chitrak (*Plumbago zeylanica*) extract was added which plays a vital role in production of anti-oxidative activity in the product (Murugan *et al.* 2018). It has also been observed that the antioxidative activity gradually declines from day 10 where similar results have been found by Chavda *et al*. (2016) with protein enriched cranberry whey beverage.

Antibiotic susceptibility: The antibiotic susceptibility of the product has been depicted on (Table 4).The product was resistant to Vancomycin, Nalidixic, Ka-

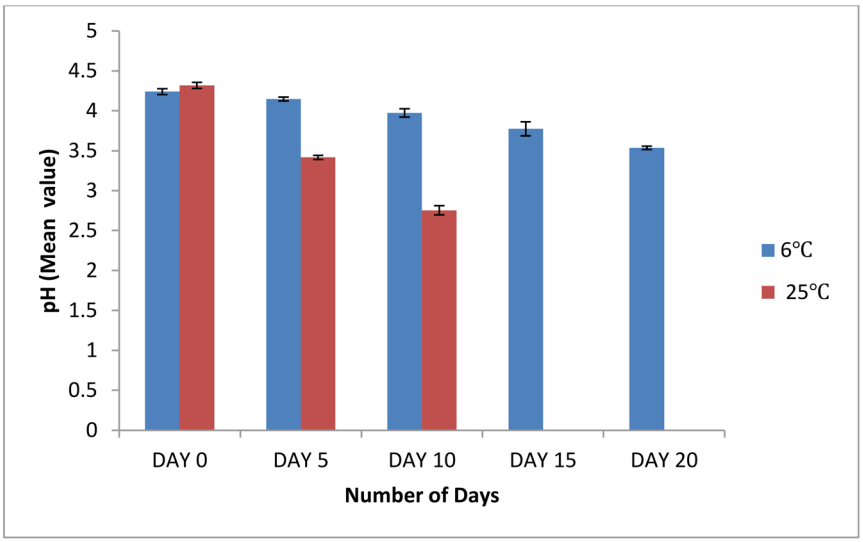

Fig 3. Shelf-life status of orange flavoured whey-based herbal beverage comparing with storage condition and storage days.Values differ significantly ($p \leq 0.05$), Mean ± SD (n = 3). .

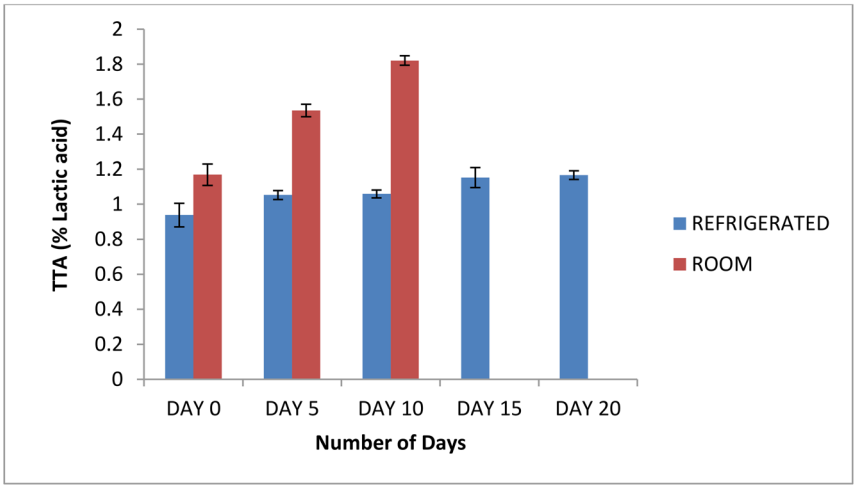

Fig 4. Shelf life status of whey-based herbal beverage comparing with storage condition and storage days.Values differ significantly ($p \leq 0.05$), Mean ± SD (n = 3).

namycin. Oxacillin and Methicillin for the samples in all the storage days and storage condition. ANOVA and Tukey's test showed significant difference among Azithromycin, Tetracycline, Streptomycin, Erythromycin and Rifampicin ($p \leq 0.05$).The LAB strains were found to be significantly sensitive to Rifampicin and Tetracycline throughout the storage period with highest susceptibility on day 20 with 40mm ZOI; Erythromycin and Azithromycin are two next most effective antibiotics. Similar results have been reported (Federici *et al*. 2014) where antibiotics such as erythromycin and tetracyclines inhibits protein synthesis which makes them susceptible to *Lactobacillus* strains (Van Hoek *et al*., 2011). On the other hand, Vancomycin, Nalidixic acid, Kanamycin, Oxacillin and Methicillin showed resistance against the product favouring the report published by Ashraf and Shah (2011).

CONCLUSION

The developed fermented whey-based herbal beverage possesses excellent sensory, antioxidative and antibiotic properties. Hence, can be an important product in the continuously developing market for functional foods. This beverage on fermentation with LAB increases the shelf life and also transforms a certain amount of lactose into lactic acid thus indicating a potential product for the lactose intolerant individuals in India. As whey is developed as a by-product of paneer and channa, its cost is assumed to be comparatively very low. Thus, it can be assessable to the lower income groups as well delivering high nutritional values. Future research is encouraged in this field concerning towards herbal whey with different medicinal plants taking account of its potential growth in the functional food market.

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